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AMERICAN
JOURNAL OF BOTANY

VOL. VIII JANUARY, 1921 NO. 1

THE FIXATION OF FREE NITROGEN BY GREEN PLANTS

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(Received for publication July 3, 1920)

The ability of chlorophyll-bearing plants to utilize the uncombined nitrogen of the atmosphere has been repeatedly investigated during the last three or four decades, and the results of numerous observations and experiments, covering a wide range of species, have been quite conflicting. In some of the earlier experiments with higher plants in pot cultures the beneficial effect of a surface layer of algae was often observed, and the ability to increase the nitrogen content of the soil by free nitrogen fixation was ascribed to members of both the blue-green (Cyanophyceae) and grass-green (Chlorophyceae) algae. Similar increases in soil-nitrogen content were observed when higher plants were excluded from the cultures, and, though bacteria were known to be present, the fixation was generally ascribed to the chlorophyll-containing forms. More recently, pure cultures of members of the Chlorophyceae have been used but the results in these cases have been almost uniformly negative. This fact, together with the discovery of widely distributed soil bacteria of the Azotobacter and Clostridium types, the ability of which to fix free nitrogen can not be questioned, has led to the belief that in impure cultures fixation is due not to the activities of the green plants but to the bacteria present in the soil. Thus it has come to be very generally accepted that members of the Chlorophyceae, as well as the higher plants, are not able to use free nitrogen.

However, the number of species which have been investigated in pure culture is small, and the culture methods employed have not always been those which are most favorable for the best growth of these organisms. Accordingly the experiments reported here were undertaken for the purpose of extending the observations over a larger number of species, grown on a variety of mineral nutrient solutions under culture conditions which would insure a rapid and vigorous growth.

LITERATURE

A complete, and in some cases detailed, review of the literature bearing on the relation of the grass-green algae (Chlorophyceae) to free nitrogen is available in a paper by Schramm (1914 *a*), so that a repetition of the account is unnecessary here. Nothing of importance relating to this subject has

[The *Journal* for December (7: 409–468) was issued January 12, 1921.]

appeared, so far as the author is aware, since the above-cited paper. As has already been indicated, the results of experiments with pure cultures have been pretty generally negative as regards the ability of these forms to increase the nitrogen content of the culture. In the light of results presented here, some of the previous experimental work will be considered in the general discussion to follow.

METHODS

Seven species of Chlorophyceae were isolated and used in pure culture. In making the isolations the plate culture method, as described by Schramm (1914 *b*), was employed. With the exception of one species, *Protococcus* sp., all isolations were made from growths occurring on soil; the material for the Protococcus culture was secured from the bark of an elm tree.

The absolute identity of all the species has not as yet been determined. Cultures have been submitted to several authorities on the group, but for some of the forms the determinations received have been somewhat at variance. For that reason no attempt has been made to apply specific names to all the organisms. Moreover, as will be apparent later, the absolute identity of the forms, though highly desirable, does not become of paramount importance because of the very similar way in which all the species seem to react. Unless otherwise indicated, therefore, the different species will be referred to by number and genus, or by number only, and as soon as more satisfactory determinations can be made a list will be published, if possible, in this journal. The forms used in the experiments include the following:

Species number 1. *Chlorella vulgaris* Beyr. There seems to be no doubt about the identity of this species.

Species number 2. *Stichococcus* sp.

Species number 3. *Protosiphon botryoides* (Kg.) Klebs.

Species number 5. *Chlorella* sp. A small form with cup-chaped chromatophore.

Species number 6. *Scenedesmus* sp.

Species number 7. *Protococcus* sp.

Species number 11. *Chlorella* sp. A large form with clathrate chromatophore.

All these species have been carried along on mineral nutrient agar for two or three years; they have been repeatedly transferred to media containing glucose or sucrose, and have frequently been examined microscopically. They are known to be free from bacteria and are pure cultures in the strict sense.

Culture Media.—In the experiments Kjeldahl flasks of Pyrex glass and of 500 cc. capacity were used as culture flasks, because of the obvious advantage of analyzing checks and cultures without transferring the material to a digestion flask. Approximately 150 grams of mineral nutrient agar were supplied as a medium for each culture. In spite of the difficulties involved in its analysis, a solid medium was chosen because of the very long-continued, vigorous growth produced on it. So far as has been observed, solution cultures, at least when unaerated, do not give a very extended or abundant development of these organisms. Since many previous experiments have also shown that these forms do not grow in pure culture in the complete absence of combined nitrogen, no attempt was made to include such cultures in the experiments.

Two experiments were performed, the first in the winter of 1917–18 and the second in the summer of 1919. In both cases the following mineral nutrient solution was employed as a standard for the preparation of the media:

NH_4NO_3	0.5 gram
$MgSO_4$	0.2 gram
K_2HPO_4	0.2 gram
$CaCl_2$	0.1 gram
$FeSO_4$	trace
Distilled water	1000 cc.

With the nitrogen content of this solution as a basis, the NH_4NO_3 was replaced in the various series of 1917–18 by glycocoll, asparagine, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$, and by urea, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ in 1919, the nitrogen content as such being approximately the same in all media. Each of these sources of nitrogen was used in duplicate series, to one of which glucose was added. (In 1919 NH_4NO_3, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ were also used in series to which mannite was added.) No change was made in the other constituents of the full nutrient solution, so that in all series these salts were present in the proportions indicated above. The 1917–18 experiment included the following series, arranged according to nitrogen sources and presence or absence of glucose:

Series 1. Glycocoll (1.07 gr. per liter)—no glucose.
Series 1*A*. Same solution, with 1 percent glucose.
Series 2. Asparagine (0.942 gr. per liter)—no glucose.
Series 2*A*. Same solution, with 1 percent glucose.
Series 3. Ammonium sulphate (0.942 gr. per liter)—no glucose.
Series 3*A*. Same solution, with 1 percent glucose.
Series 4. Ammonium nitrate (0.5 gr. per liter)—no glucose.
Series 4*A*. Same solution, with 1 percent glucose.
Series 5. Calcium nitrate [$Ca(NO_3)_2.4H_2O$, 1.475 gr. per liter]—no glucose.
Series 5*A*. Same solution, with 1 percent glucose.

In making up the media for the above series, sufficient nutrient solution with any one nitrogen source was prepared to supply both the series without glucose and the series with glucose. For these solutions the required amounts of the several constituents, with the exception of ferrous sulphate, were weighed out individually and dissolved in the proper volume of distilled water. (A stock solution of ferrous sulphate was prepared by dissolving

0.1 gr. in 2,000 cc. of distilled water, 50 cc. of which were used in the preparation of each liter of nutrient solution.) The solution thus prepared was divided into two equal quantities in large flasks, 1.5 percent agar being added to each, and 1 percent glucose to one portion. The total nitrogen content of the medium with any one nitrogen source should therefore be the same per unit weight in the two series, with and without glucose, except for traces of nitrogen in the glucose or for slight discrepancies in the actual amount or composition of agar added. The nitrogen-containing compounds were not dried to constant weight nor was the agar purified in any way, all substances being added to the solution directly from the stock bottles. The chemicals used were Baker's "analyzed" and Merck's "highest purity"; the agar was of the kind known as "Difco bacto."

The total nitrogen content of each culture medium was determined by actual analysis of weighed portions of that medium. It is obvious that by this method any nitrogen introduced with the agar or as impurities with the glucose would be completely accounted for.[1]

The 1919 experiment was a partial duplication and an extension of that of the previous year and included the following series:

Series 6. Urea (0.375 gr. per liter)—without glucose.
Series 6*A*. Samė solution, with 1 percent glucose.
Series 7*A*. Ammonium sulphate (0.621 gr. per liter), with 1 percent glucose.
Series 7*B*. Ammonium sulphate (as above), with 1 percent mannite.
Series 8. Ammonium nitrate (0.5 gr. per liter)—no glucose or mannite.
Series 8*A*. Same solution, with 1 percent glucose.
Series 8*B*. Same solution as series 8, with 1 percent mannite.
Series 9. Calcium nitrate [$Ca(NO_3)_2.4H_2O$, 1.475 gr. per liter]—no glucose or mannite.
Series 9*A*. Same solution, with 1 percent glucose.
Series 9*B*. Same solution as series 9, with 1 percent mannite.

Each complete solution was placed in the autoclav under 15 pounds' pressure until the agar was dissolved. The solution was then filtered through absorbent cotton; the filtrate was free from sediment.

Introduction of the Agar.—The Kjeldahl flasks were cleaned in the usual way and dried in the hot air oven. On removal from the oven, cotton plugs were inserted in the mouths of the flasks to prevent the entrance of dust. Each flask was numbered by means of a carborundum point and weighed to within 0.05 gram on a Mackenzie one-pan balance, the cotton plug being removed only during the weighing. The flasks were then stored in clean, dry boxes until required.

As soon as the medium for a series was prepared the required number of flasks (11 in 1917–18, 24 in 1919) were arranged in one of the special wooden racks (see fig. 1, Plate I) and 150 cc. of the hot agar solution was added to each flask. The flasks were then immediately weighed in the order in which the agar was introduced. The mouths of the flasks remained plugged with cotton except during the actual processes of introducing the agar and of weighing. Although water vapor condensed on the walls and necks of flasks as the agar cooled, the use, with a number of flasks, of rubber stoppers instead of cotton plugs, demonstrated that there was no detectable loss of water vapor through the cotton plug during the interval between the introduction of the agar solution and the weighing. Thus the actual weight of medium in each flask was known, and, as will be seen from the tables, differences resulted of one or two grams in the weight of approximately equal volumes between the first and last flask of a series to receive the medium, due to the cooling of the agar during the processes involved.

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[1] Numerous analyses of the agar showed the nitrogen content to be about 1 mg. for the amount present in each culture flask. It will be noticed that the analyses for the total nitrogen of the media may not have yielded exactly the calculated amounts, because of the moisture present in the nitrogen-containing compounds.

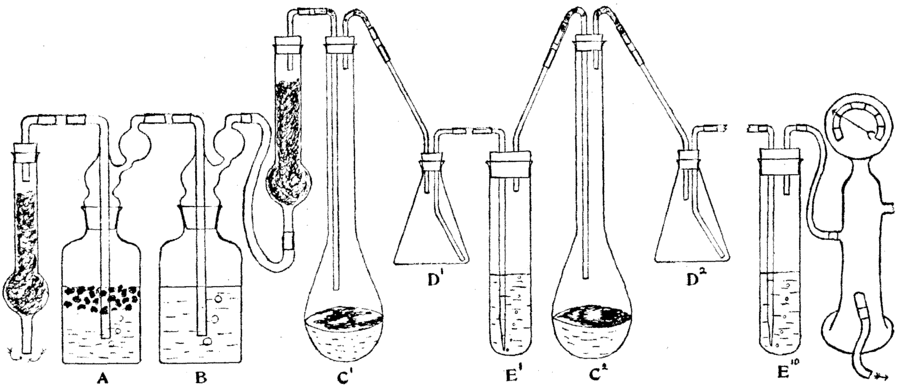
After the second weighing each flask was provided with a two-hole rubber stopper carrying intake and outlet glass tubes, the outer arms of these tubes being adjusted so as to be readily connected in series by means of rubber tubing. Long cotton plugs were loosely adjusted in the bore of each outer arm. The flasks were sterilized at 15 pounds' pressure for 20 minutes, the stoppers resting lightly in the mouths of the flasks but being tightly adjusted upon removal from the autoclav, the hands being moistened with alcohol for this operation. The flasks were allowed to cool in a dust-proof case.

Inoculation.—The inoculations were made in the laboratory under a glass dust shield open on one side only. The inoculum consisted of a suspension of the algal cells in a test tube of sterilized nutrient solution minus combined nitrogen. Special cultures on hard (2 percent) agar were prepared, so that in making the suspension no agar was introduced with the cells. The tube was thoroughly shaken to secure a uniform suspension of the inoculum, of which ten drops were added to each flask in the 1917–18 experiment and one cubic centimeter was similarly added in 1919. In the former experiment four species were used and two flasks in each series were inoculated with the same species, three flasks of each medium remaining uninoculated as checks. In 1919 seven species were used, three flasks of each series being inoculated with each species with the exception of species no. 3 and no. 7, in which cases only two flasks of any one medium were inoculated; three flasks of each series remained uninoculated as checks.

After the rubber stoppers were tightly fitted in the flasks, melted paraffin was run in around the flared neck, and the stopper and a portion of the neck of each flask were covered with sterilized cotton. During the two experiments eighteen contaminations occurred out of a total of 340 flasks.

Aeration.—The intake and delivery tubes of the flasks of each series were connected with rubber tubing for the purpose of aeration. In making connections the free ends of the glass tubes were painted with 95 percent alcohol, which was used also in washing out the bore of the rubber tubing.

In setting up the first experiment it was thought that in the process of aeration a loss of nitrogen from the medium in the form of ammonia might occur, especially in view of the fact that the medium was slightly alkaline, so that a tube of acid was inserted in the series just beyond each culture flask. For this purpose large test tubes, 200 x 25 mm., and containing 25 cc. of standardized N/10 sulphuric acid were used. As a precaution against the backflow of this acid into the cultures, small Erlenmeyer flasks of 180 cc. capacity were placed between each culture flask and its corresponding acid tube. The arrangement of the apparatus can readily be understood by consulting text figure 1. Two gas-washing bottles, *A* and *B*, were placed at the head of each series; *A* contained 30 percent sulphuric acid and a quantity of pumice stone; *B* contained sterilized distilled water. Air entered the series through a calcium chloride tube filled with cotton, was washed free of ammonia by the acid in *A* and was moistened by the water in *B*. Oxides of nitrogen would also be removed by the water. Before entering the culture flask C' the air passed through a second calcium chloride tube containing sterilized cotton. The intake tube of each culture extended to within an inch or so of the surface of the agar medium, whereas the delivery tube merely penetrated the rubber stopper, so that in the process of aeration the air above the agar surface was completely changed. After leaving the culture flask, the air passed through the safety flask D' and bubbled through the acid in the adjoining test tube E'. The intake tube of the latter was drawn out to a fine point which extended to the very bottom of the test tube, so that only very small bubbles were formed, insuring a thorough washing of the air before it passed into the next culture flask of the series. The delivery tube of the last acid tube in the series was connected to a filter pump, by means of which the air was drawn through the whole series at once.



TEXT FIG. 1. Detail of a portion of one of the series of 1917–18. Explanation in the text.

When the ten series of the first experiment had been completely assembled, the delivery tubes at the end of each rack were connected in a single series by means of T-tubes so that aeration of all ten series could be accomplished by one operation. Each of these delivery tubes was provided with a screw clamp which was kept tightly closed except during the process of aeration, when it served to control the volume of air passing through the series. With a few exceptions aeration was continued for an hour every morning, it being considered that this would entirely replace the air in the apparatus. The 1917–18 experiment as completely assembled is shown in figure 1, Plate I.

At the end of the first experiment, titrations of the contents of the acid tubes showed that no appreciable change had taken place in the concentration in any instance. It was assumed, therefore, that with these species and with the conditions realized in the experiment there was no loss of ammonia from the culture flasks. For this reason the tubes of acid were omitted from the second experiment, and in the process of aeration the air passed directly from one culture flask to the next in the series. Because of the expansion of the air in the culture flasks during the hot summer days, the liquids in the gas-washing bottles were frequently forced out through the intake tubes of these bottles,—making it necessary to place safety flasks outside the acid bottle, and between the acid and water bottles, to receive those liquids. During the process of eration the acid and water were drawn back into the proper bottles so that no air ever entered the series without first passing through the liquids. Aeration was continued for an hour every other morning during the growing period.

Cultural Conditions.—Soon after the inoculation of the culture flasks the ten racks were transferred to the greenhouse where more uniform conditions of light and temperature prevailed than in the laboratory. Since preliminary tests showed that agar cultures of the organisms used were soon killed by direct sunlight, the bench occupied by the apparatus was covered with a canopy of black cloth, which reduced the actinic light intensity to about one eighth that of the normal greenhouse illumination. On cloudy days, however, this canopy was rolled up on both sides, thus permitting better illumination; on clear days the west side was open during the morning only, and during the afternoon the east side only was exposed. The arrangement of the apparatus as finally assembled in the greenhouse is shown in figure 2, Plate I, which is a photograph of the 1919 experiment.

GROWTH OF THE CULTURES

Length of Growing Period.—The approximate length, in days, of the growing period of each series is indicated in the headings of the tables which follow. Inoculations in the first experiment were completed on August 31, 1917, and the analyses were begun in April, 1918. The final inoculations of the second experiment were made in May, 1919, and analyses were started in November of the same year.

Method of Recording Growth.—Records of the growth of the cultures were made at intervals of three or four weeks. These consisted of written notes comparing the development of the different species on the same medium, and the difference in amount of growth of the same species on the ten different media. Charts were also prepared at about monthly intervals showing the growth in each culture flask by means of colored crayons. These were found very helpful in making growth comparisons, as they present to the eye at once the relative development in every flask.

Since it was the plan of the experiments to analyze the entire contents of the culture flasks at the end of the growing period, it was not found advisable to attempt any actual weight determinations of the "crop" produced on the various media by the different species. Experiments with solution cultures are in progress now from which it is hoped some accurate data may be secured, showing the actual amounts of growth produced on different media and what relation the dry weight of algal material produced bears to free nitrogen assimilation. From a comparison of the written notes and colored charts, however, the following general statements may be made.

1917–18 Experiment.—In this experiment there was a remarkable similarity in the amount of development of all four species on any one of the media used. Only in a few cases did there appear to be any marked specific differences in the reactions of the organisms to the medium. In general, the presence of glucose resulted in a vigorous and rapid development of all species, irrespective of the nitrogen source.

Series 1–3. The relative growth of all the cultures is indicated in the tables which follow, by means of plus signs. Since no fixation occurred in the series in which the nitrogen was supplied as ammonium sulphate or in the organic forms used, the detailed observations of these series are omitted. The results on the nitrate media, however, were so striking that a more detailed account of the growth on these media is here presented.

Series 4 (ammonium nitrate, without glucose). Growth was slow but steady in all cases. Species nos. 1, 5, and 6 continued healthy to the last, giving "very fair" growths. The growth of species no. 2 was "fair," but the cultures were dead at the end of the experiment.

Series 4A (ammonium nitrate, with 1 percent glucose). All species started with very vigorous growths, the effect of the presence of glucose being very evident. Species no. 1 produced a "luxuriant" growth at first, but after three months began to deteriorate, turning brown over most of the surface. Before growth had completely ceased, however, both cultures of this species began to revive, and by the end of the fourth month were again bright green. The cultures then slowly waned a second time, only small portions remaining green at the end of the experiment. Species nos. 2, 5, and 6 grew steadily from the start and remained healthy, no. 2 producing a "luxuriant" growth and nos. 5 and 6 "very good" growths.

Series 5 (calcium nitrate, without glucose). The growth was very slow with all species, but all remained healthy. Total growth of species no. 2 was "fair," while for nos. 1, 5, and 6 it was "very fair."

Series 5A (calcium nitrate, with 1 percent glucose). All four species gave vigorous growths on this medium, continuing healthy to the end of the experiment. The effect of the presence of glucose was apparent from the start. The growth of species no. 2 was "luxuriant" and appeared slightly better than the others, all of which were "very good."

1919 Experiment.—So far as this experiment duplicated the previous one, the same general type of growth resulted. The presence of glucose in the medium markedly stimulated the development of all species, irrespective of the source of combined nitrogen. It is also true, however, that death of the cultures always occurred first on the media containing glucose. The presence of mannite apparently had no effect by way of increasing the rapidity or amount of growth of any of the species on any of the three media to which this compound was added. The amount of growth produced on these media appeared practically the same as produced by the same organism with the same source of nitrogen but without either glucose or mannite.

Series 6 and 7. As in the previous experiment, no fixation occurred where combined nitrogen was supplied in an organic form or as ammonium sulphate; growth observations for these media are therefore omitted.

Series 8 (ammonium nitrate, without glucose or mannite). A slow, steady growth resulted, as in the previous experiment. At analysis all cultures were healthy. Species nos. 1, 6, and 11 produced "very fair" growths; nos. 2 and 5, "fair." Species nos. 3 and 7 were not grown on this medium.

Series 8A (ammonium nitrate, with 1 percent glucose). All species grew very vigorously at first, but deterioration soon set in and by the end of one month all cultures of nos. 1 and 2 were dead, after a "fair" growth, and species nos. 3 and 6 were rapidly waning. The cultures of no. 3 died after a "fair" growth. One culture of no. 6 also died, but the two others revived and remained healthy to the end of the experiment, giving "very good" growths. Species no. 11 gave a "luxuriant" growth, but at the end of the experiment was turning brown. Nos. 5 and 7 remained healthy throughout the growing period, both producing "very good" growths.

Series 8B (ammonium nitrate, with 1 percent mannite). Growth on this medium was slow, and in general very much as in series 8. All cultures remained healthy at the end of the experiment. Species nos. 1, 3, 6, and 11 gave "very fair" growths, the development being somewhat better than with species nos. 2, 5, and 7.

Series 9 (calcium nitrate, without glucose or mannite). Growth was very slow, and strikingly like that in series 8. All cultures remained healthy, species no. 6 giving a "good" growth, nos. 1 and 11 "very fair" growths, and nos. 2 and 5 "fair." Species nos. 3 and 7 were not included in this series.

Series 9A (calcium nitrate, with 1 percent glucose). Development was very vigorous from the start, all cultures soon producing a complete coat over the agar surface. At the end of a month, species nos. 3, 5, 6, and 11 began to turn yellow. The deterioration, however, did not progress far, and after another month or two all cultures were again green, remaining healthy until analysis. Species nos. 1, 2, and 7 remained healthy throughout the entire growing period. At the end of the experiment a "luxuriant" growth had resulted with species nos. 2 and 11, "very good" growth with nos. 1, 5, and 6, "good" with no. 3, and "slight" with no. 7.

Series 9B (calcium nitrate, with 1 percent mannite). The development was very similar to that in series 9, being slow but steady. All cultures remained healthy to the end. Species nos. 1 and 11 produced "very fair" growths, nos. 2, 5, 6, and 3 "fair," and no. 7 only "slight."

It should be noted that in the 1917–18 experiment the development of the four species on the two nitrate media with glucose (series 4*A* and 5*A*) was very similar, the growth being either "luxuriant" or "very good" in all cases. Of the two, however, the cultures of series 5A appeared somewhat the better. In striking contrast with this condition was the growth of the various species on similar media of the 1919 experiment (series 8*A* and 9*A*). The species which gave such good growths on ammonium nitrate with glucose (series 4*A*) in 1917–18 showed scarcely any development on this medium in 1919 (series 8*A*), with the possible exception of species no. 5. However, on calcium nitrate with glucose (series 9*A*) the growth of these species was practically the same as was secured on the similar medium of 1917–18 (series 5*A*). Even though a few of the flasks of series 8*A* were reinoculated, the growth continued poor; likewise, "reserve" flasks of this medium which were not introduced in the series at the beginning of the experiment gave very similar growths of species nos. 1 and 2 when inoculated a few months before the end of the growing period. This difference in the amount of growth produced in the two experiments may possibly be related to the difference in the seasons during which the experiments were conducted.

Analyses

At the end of a growing period of from six to eight months the cultures and checks were analyzed for total nitrogen content. After making a final record of the growth and condition of the cultures, a small loop of the algal material from each flask was transferred to a drop of sterilized nutrient solution and examined microscopically. In 1918 transfers were also made from each culture to nutrient agar containing 1 percent glucose, and to the same medium made acid by the addition of 1 percent hydrochloric acid. Two tubes of each of these media were inoculated from each culture flask, but in no case did contaminations appear that were not apparent from the microscopical examination. This precaution was therefore omitted in 1919, the microscopical examination being relied upon to detect contaminations not apparent to the naked eye. In all cases, however, such contaminations as occurred were perfectly evident from macroscopic examinations, the majority of them appearing soon after inoculation.

After considerable preliminary work with total nitrogen determinations, the Gunning-Kjeldahl[1] method was adopted for media free from nitrates, and in the presence of nitrates the Förster modification of this method was used. In the former method the digestion mixture consists of a solution of 20 grams phosphorus pentoxide (P_2O_5) in 500 cc. sulphuric acid of sp. gr. 1.84, 20 cc. of this solution being added to each culture flask. After the addition of 10 grams potassium sulphate to each, the flasks were heated slowly over a low flame. The presence of the agar made the digestions particularly trying, as it was necessary to watch the flasks constantly at the beginning of the process in order to prevent the contents from foaming up into the necks. It was found advisable to start with a very low flame and to shake the flasks occasionally until sufficient agar had gone into solution to allow the ready escape of bubbles from below. At this point the full flame was used, and the water boiled off vigorously until foaming began. The flame was then turned very low again for about 30 minutes or until the appearance of dense, white fumes, at which time foaming gradually ceased. The fire was then slowly increased to full capacity and the digestion continued for 15 or 20 minutes after a clear liquid resulted. About 1½ hours were required for digestion after foaming ceased. In all cases the flasks at the end of the digestion were perfectly clean and the liquid was entirely transparent.

The distillation was carried out in the usual way. About 150–200 cc. of distilled water was added to each flask when cool, the neck of the flask being thoroughly washed down in the process. The solution was made alkaline with 50 cc. concentrated sodium hydroxide, and, after the addition of a gram of granulated zinc, the flask was immediately connected to the still. The ammonia was distilled over through block tin tubes into a 500-cc. Erlenmeyer flask containing 30 cc. standardized tenth-normal sulphuric acid, diluted with enough distilled water to cover completely the end of the delivery tube. Standard traps were used between the Kjeldahl flask and the condenser. Distillation was continued until the contents of the flask began to "bump." About 125 cc. of water was distilled over in this process, and it was usually completed in about 40 minutes. During the latter part of the distillation the receiving flask was drawn away from the still sufficiently to uncover the end of the delivery tube, the inside walls of which were washed down by the remaining distillate. At the end of the distillation the delivery tube, as well as the inside wall of the receiving flask, was washed down with a small amount of distilled water. The excess acid was titrated against tenth-normal sodium hydroxide, using cochineal as an indicator.

The Förster modification, employed in analyzing the media containing nitrates, consists essentially in the addition of sodium thiosulphate to the digestion mixture. The procedure was as follows: A considerable part of the water of the medium was boiled off in the presence of 10 cc. of concentrated sulphuric acid, the process being continued until active foaming began. When cool, 20 cc. of the phenol-sulphuric acid digestion mixture (100 gr. phenol in 924 cc. concentrated sulphuric acid) were added and allowed to stand several hours or over night, the mouths of the flasks being covered during this time. Two grams of sodium thiosulphate were then added, and, after the reaction was completed, 1 gram of mercuric oxide, 1 gram of zinc dust, 10 cc. of concentrated sulphuric acid, and finally 10 grams of potassium sulphate were added in the order named. The flasks were heated over a low flame until foaming ceased, when the flame was gradually increased to full intensity. Digestion was continued for about 20 minutes after the liquid became clear, the process requiring about 2½ or 3 hours. The distillation was carried out as with the Gunning method, except that before making the solution alkaline the mercury was precipitated out by the addition of 25 cc. potassium sulphide solution (20 gr. K_2S in 500 cc. H_2O).

Numerous "blank" determinations of the nitrogen content of the reagents used in the two methods were made, the average of these being deducted from the determinations of the cultures and checks. Care was taken to use uniform reagents for the analyses of each series.

In the preliminary determinations no difficulty was experienced with the Gunning method in securing complete recovery of the nitrogen from solutions of urea or ammonium sulphate. In the presence of agar or of agar and glucose the usual trouble with foaming was encountered, but the determinations checked readily within the limits of experimental error.

The recovery of total nitrogen, including nitrates in the presence of agar, was more difficult, but consistent results were obtained with the Förster method as outlined above. It was found necessary to allow the digestion mixture to stand a considerable length of time (usually over night) in contact with the dissolved and partially concentrated agar medium before the addition of the other reagents. Following the experience of Duggar and Davis (1916), it was also found advisable to permit the flasks to stand about an hour after the addition of the sodium thiosulphate. The low results obtained when the digestions are completed more quickly may possibly be due to the rather extensive dilution of the reagents by the water of the culture medium and the consequent slower reduction of the nitrates. Tables A and B show some of the results obtained in the preliminary determinations.

TABLE A. *Recovery of total nitrogen from ammonium nitrate by Förster method*
Solution = 1.4285 grams dry NH_4NO_3 in 250 cc. distilled water

10 Cc. Solution Made Alkaline and Distilled	Mg. N as NH_3 Recovered	Mg. N as NH_3 by Calculation	Mg. Difference
1	9.980	10.000	−0.020
2	9.896	10.000	−0.104
3	9.980	10.000	−0.020
10 Cc. Solution Digested by Föster Method	**Mg. Total Nitrogen Recovered**	**Mg. Total Nitrogen by Calculation**	**Mg. Difference**
1	19.812	20.000	−0.198
2	19.540	20.000	−0.460
3	19.931	20.000	−0.069

TABLE B. *Recovery of total nitrogen from ammonium nitrate in the presence of agar agar by Förster method*

Solution of NH_4NO_3. Total calculated N in 10 cc. = 26.252 mg.

Total Mg. N Found in 10 Cc. Solution	Total Mg. N Found in 10 Cc. Solution Plus 2 Grams Agar	Mg. N Found in 2 Grams Difco Agar	Mg. N Recovered
26.268	27.760	1.826	25.993
26.219	27.372	1.741	25.615
26.024	26.994	1.703	25.237

It is apparent from table B that the agar interferes somewhat with the complete recovery of nitrogen. However, as the amount of agar in all series was the same in both checks and cultures, the results, though perhaps low, are still strictly comparable.

RESULTS

The results of the analyses of the cultures are presented in tables 1–9B. These are numbered to correspond with the series, each series representing a single medium. As already explained, the only constituent of the mineral nutrient solution which was varied in the different media was the nitrogen source; the latter is indicated in the heading of each table. The presence or absence of glucose or mannite is indicated in the sub-heading. In the third column the relative growth in each culture flask is shown by means of plus marks; in nearly all cases the growth in the two or three flasks of a series inoculated with the same organism was practically the same, but a few exceptions may be noted. In the fourth column is given the total nitrogen (in milligrams) found in each flask. This figure represents the total nitrogen calculated from the titration, minus the average amount of nitrogen in the blanks. The other columns are self-explanatory.

TABLES 1 AND 1A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1917–1918. Growth period, 230 days. Nitrogen source,* GLYCOCOLL

1. *Medium without glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
–	Check	Con.*	32.655	145.90	22.382	22.214	
–	Check	Ster.	32.389	147.10	22.018		
–	Check	Ster.	32.796	147.45	22.242		
1	*Chlorella vulgaris* Beyr.	+++†	31.823	144.90	21.962	22.017	−0.197
1		+++	32.036	145.15	22.071		
2	*Stichococcus* sp.	++++*	31.859	145.30	22.926	22.020	−0.194
2		+++	32.230	145.75	22.113		
5	*Chlorella* (?) sp.	+++	31.965	146.15	21.871	22.028	−0.186
5		+++*	32.478	146.40	22.184		
6	*Scenedesmus* (?) sp.	+++	32.354	146.70	22.055	21.930	−0.284
6		+++	32.053	147.00	21.805		
	Average all cultures		.....	.....	.....	21.999	−0.215

1A. *Same medium, with 1 percent glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
–	Check	Ster.	31.646	146.70	21.572	21.502	
–	Check	Ster.	31.841	148.00	21.514		
–	Check	Ster.	31.735	148.15	21.421		
1	*Chlorella vulgaris* Beyr.	+++++	31.010	146.55	21.160	21.466	−0.056
1		+++++	31.859	146.60	21.732		
2	*Stichococcus* sp.	+++++	31.438	146.55	21.452	21.450	−0.052
2		+++++	31.452	146.65	21.447		
5	*Chlorella* (?) sp.	+++++	30.656	147.20	20.826	21.055	−0.447
5		+++++	31.328	147.20	21.283		
6	*Scenedesmus* (?) sp.	+++++	31.540	147.50	21.383	21.378	−0.124
6		+++++	31.611	147.90	21.373		
	Average all cultures		.....	.....	.....	21.332	−0.170

TABLES 2 AND 2A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1917–1918. Growth period, 234 days. Nitrogen source,* ASPARAGINE

2. *Medium without glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
–	Check	Ster.	27.808	146.50	18.982	19.031	
–	Check	Ster.	28.162	147.25	19.125		
–	Check	Ster.	27.967	147.30	18.987		
1	*Chlorella vulgaris* Beyr.	++	27.861	146.20	19.057	18.966	−0.065
1		++	27.614	146.30	18.875		
2	*Stichococcus* sp.	++	28.410	146.30	19.419	19.192	+0.161
2		++	27.755	146.35	18.965		
5	*Chlorella* (?) sp.	+++	27.667	146.50	18.885	18.806	−0.225
5		+++	27.454	146.60	18.727		
6	*Scenedesmus* (?) sp.	+++	27.614	146.90	18.798	18.783	−0.248
6		+++	27.578	146.95	18.767		
	Average all cultures		.....	.....	.....	18.937	−0.094

* Checks or cultures which were contaminated are designated with an asterisk.

† Relative growth of the cultures is indicated by plus signs, as follows: + = "slight," ++ = "fair," +++ = "very fair," ++++ = "good," +++++ = "very good," ++++++ = "luxuriant."

2A. *Same medium, with 1 percent glucose*

–	Check	Ster.	27.260	147.05	18.538	18.655	
–	Check	Ster.	Lost	147.70	.....		
–	Check	Ster.	27.773	147.95	18.772		
1	*Chlorella vulgaris* Beyr.	++++++	26.924	146.95	18.322	18.344	−0.311
1		++++++	26.941	146.70	18.365		
2	*Stichococcus* sp.	++++++	27.277	146.80	18.581	18.443	−0.212
2		++++++	26.888	146.90	18.304		
5	*Chlorella* (?) sp.	++++++	26.959	147.25	18.308	18.119	−0.536
5		++++++	26.393	147.20	17.930		
6	*Scenedesmus* (?) sp.	++++++	26.835	147.40	18.205	18.289	−0.366
6		++++++	27.154	147.80	18.372		
	Average all cultures		.....	.....	.....	18.299	−0.356

TABLES 3 AND 3A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1917–18. Growth period, 226 days. Nitrogen source,* AMMONIUM SULPHATE

3. *Medium without glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
–	Check	Ster.	29.842	146.75	20.335	20.441	
–	Check	Ster.	30.338	147.65	20.547		
1	*Chlorella vulgaris* Beyr.	+++	29.719	145.50	20.425	20.388	−0.053
1		+++	29.701	145.95	20.350		
2	*Stichococcus* sp.	++	30.055	145.80	20.614	20.591	+0.150
2		++	30.090	146.30	20.567		
5	*Chlorella* (?) sp.	++	30.055	147.00	20.445	20.658	+0.217
5		+++*	30.691	147.05	20.871		
6	*Scenedesmus* (?) sp.	+++	30.090	147.20	20.441	20.494	+0.053
6		+++	30.284	147.40	20.546		
	Average all cultures		.....	.....	.....	20.533	+0.092

3A. *Same medium, with 1 percent glucose*

–	Check	Ster.	29.400	147.10	19.986	19.957	
–	Check	Ster.	29.233	148.05	19.739		
–	Check	Ster.	29.825	148.05	20.145		
1	*Chlorella vulgaris* Beyr.	(+++++)†	28.834	146.75	19.648	19.657	−0.300
1		(+++++)	28.869	146.80	19.666		
2	*Stichococcus* sp.	(+++++)	29.365	146.85	19.996	19.836	−0.121
2		(+++++)	28.923	147.00	19.675		
5	*Chlorella* (?) sp.	+++++++	29.188	147.35	19.809	19.776	−0.181
5		+++++++	29.082	147.30	19.743		
6	*Scenedesmus* (?) sp.	(+++)	28.746	147.75	19.456	19.261	−0.696
6		(+++)	28.180	147.80	19.066		
	Average all cultures		.....	.....	.....	19.634	−0.323

† Plus signs enclosed in parentheses indicate that the culture was dead at the time of analysis.

TABLES 4 AND 4A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1917–18. Growth period, 270 days. Nitrogen source,* AMMONIUM NITRATE

4. *Medium without glucose*

Culture No.	Culture Species	Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.	Gain Percent
–	Check	Ster.	23.569	146.90	16.044	15.788		
–	Check	Con.*	23.885	147.95	16.144			
–	Check	Con.*	22.445	148.25	15.175			
1	*Chlorella vulgaris*	+++*	22.410	146.20	15.328	15.758	−0.030	
1	Beyr.	+++*	23.674	146.25	16.188			
2	*Stichococcus* sp.	(++)	24.921	146.45	17.017	16.542	+0.754	4.7
2		(++)	23.569	146.70	16.066			
5	*Chlorella* (?) sp.	++++	25.729	146.90	17.515	17.027	+1.239	7.8
5		+++	24.377	147.40	16.538			
6	*Scenedesmus* (?) sp.	+++	25.940	147.60	17.574	17.574	+1.786	11.3
6		++	Lost	147.75	.....			
	Average all cultures		.....	.....	.....	16.725	+0.937	5.9

4A. *Same medium, with 1 percent glucose*

Culture No.	Culture Species	Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.	Gain Percent
–	Check	Ster.	24.323	147.75	15.794	16.032		
–	Check	Ster.	23.446	148.60	15.778			
–	Check	Ster.	24.095	145.90	16.525			
1	*Chlorella vulgaris*	(++++)	32.747	146.90	21.978	22.136	+6.104	38.
1	Beyr.	(+++)+	32.817	147.20	22.294			
2	*Stichococcus*	++++++	32.269	147.10	21.987	21.910	+5.878	36.
2		++++++	32.203	147.50	21.832			
5	*Chlorella* (?) sp.	+++++	33.291	147.90	22.509	22.394	+6.362	39.6
5		+++++	33.028	148.25	22.278			
6	*Scenedesmus* (?) sp.	+++++	33.624	148.25	22.681	22.179	+6.147	38.3
6		+++++	32.168	148.40	21.676			
	Average all culcultures........		.....	.....	.....	22.155	+6.123	38.

TABLES 5 AND 5A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1917–18. Growth period, 297 days. Nitrogen source,* CALCIUM NITRATE

5. *Medium without glucose*

Culture No.	Culture Species	Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.	Gain Percent
–	Check	Ster.	24.850	147.35	(16.903	†16.903		
–	Check (Loss)	Ster.	21.182	148.25	14.289)			
1	*Chlorella vulgaris*	+++	23.797	145.65	16.338	16.258	−0.645	
1	Beyr.	+++	23.586	145.80	16.177			
2	*Stichococcus* sp.	++	23.990	146.05	16.425	16.368	−0.535	
2		++	23.902	146.55	16.310			
5	*Chlorella* (?) sp.	+++	27.219	147.30	18.479	18.755	+1.852	11.
5		+++	28.079	147.55	19.030			
6	*Scenedesmus* (?) sp.	+++	27.079	147.80	18.322	16.565	−0.338	
6		+++	24.078	148.00	16.269			
6		+++	23.393	148.25	15.105			
	Average all cultures		.....	.....	.....	16.987	+0.084	

5A. *Same medium, with 1 percent glucose*

–	Check (Loss)	Ster.	22.603	148.50	(15.221)	†16.903		
–	Check (Loss)	Ster.	18.360	147.40	(12.456)			
1	*Chlorella vulgaris* Beyr.	+++++	35.274	147.05	23.988	24.430	+7.527	45.1
1		+++++	36.572	147.05	24.871			
2	*Stichococcus* sp.	++++++	36.029	147.25	24.468	23.604	+6.701	39.6
2		++++++	33.519	147.40	22.740			
5	*Chlorella* (?) sp.	+++++	35.327	147.80	24.402	24.402	+7.499	44.3
5		+++++	Lost	147.90	.....			
6	*Scenedesmus* (?) sp.	+++++	35.502	148.25	23.947	23.956	+7.053	41.7
6		+++++	34.607	148.80	23.257			
6		++++	36.590	148.35	24.665			
	Average all cultures		.....	.....	.....	24.098	+7.195	42.5

TABLES 6 AND 6A. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1919. Growth period, 165 days. Nitrogen source,* UREA

6. *Medium without glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg, Loss or Gain, 100 Gr.
No.	Species						
—	Check	Ster.	25.212	148.05	17.029	17.465	
—	Check	Ster.	26.315	148.30	17.744		
—	Check	Ster.	26.161	148.45	17.623		
1	*Chlorella vulgaris* Beyr.	+++	24.807	148.15	16.745	16.696	−0.769
1		+++	24.779	148.15	16.726		
1		+++	24.583	147.95	16.616		
2	*Stichococcus* sp.		24.667	148.05	16.661	16.714	−0.715
2			24.988	148.05	16.878		
2			24.580	148.05	16.603		
5	*Chlorella* (?) sp.		25.212	148.05	17.029	16.737	−0.728
5			24.779	148.15	16.726		
5			24.388	148.20	16.456		
6	*Scenedesmus* (?) sp.	++	24.480	148.15	16.524	16.831	−0.634
6		++	24.960	148.25	16.836		
6		++	25.407	148.30	17.132		
11	*Chlorella* (?) sp.	++	25.687	148.20	17.333	17.199	−0.266
11		++	25.533	148.30	17.217		
11		++	25.282	148.30	17.048		
	Average all cultures		.....	.....	.....	16.835	−0.630

† Because of the use of some old Jena flasks in completing Series 5 and 5A, a number of determinations of checks and cultures were lost. Accordingly, the highest complete check determination is taken for the nitrogen content of the media of these two series.

6A. *Same medium, with 1 percent glucose*

—	Check	Ster.	27.348	148.40	18.429	18.056	
—	Check	Ster.	26.203	148.50	17.645		
—	Check	Ster.	26.943	148.90	18.095		
1	*Chlorella vulgaris*	+++++	26.301	148.35	17.729	17.783	−0.273
1	Beyr.	+++++	26.231	148.40	17.676		
1		+++++	26.622	148.35	17.945		
2	*Stichococcus* sp.	++++	25.882	148.45	17.435	17.426	−0.630
2		++++	25.645	148.45	17.275		
2		++++	26.064	148.35	17.569		
5	*Chlorella* (?) sp.	+++++	25.756	148.35	17.366	17.590	−0.466
5		+++++	26.762	148.40	18.034		
5		+++++	25.784	148.45	17.369		
6	*Scenedesmus* (?) sp.	+++++	26.580	148.50	17.899	18.226	+0.170
6		+++++	27.390	148.45	18.451		
6		+++++	27.209	148.45	18.329		
11	*Chlorella* (?) sp.	+++++	26.860	148.45	18.094	18.295	+0.239
11		+++++	27.363	148.45	18.433		
11		+++++	27.306	148.75	18.357		
7	*Protococcus* sp.		26.846	148.70	18.054	18.406	+0.350
7			27.893	148.70	18.758		
3	*Protosiphon*	+++++	27.069	148.70	18.204	18.258	+0.202
3	*botryoides*	+++++	27.265	148.90	18.311		
	Average all cultures		.....	.....	.....	17.998	−0.058

TABLES 7A AND 7B. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1919. Growth period, 175 days. Nitrogen source,* AMMONIUM SULPHATE

7A. *Medium with 1 percent glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
—	Check	Ster.	21.385	148.30	14.421	14.462	
—	Check	Ster.	21.986	148.80	14.776		
—	Check	Ster.	21.190	149.35	14.188		
1	*Chlorella vulgaris*	(+)	20.729	148.40	13.968	14.084	−0.378
1	Beyr.	(+)	20.799	148.15	14.039		
1		(+)	21.148	148.45	14.246		
2	*Stichococcus* sp.	(+++)	20.561	148.25	13.869	14.176	−0.286
2		(+++)	21.651	147.95	14.634		
2		(+++)	20.799	148.30	14.025		
5	*Chlorella* (?) sp.	+++++	20.380	148.60	13.715	13.925	−0.537
5		+++++	20.771	148.65	13.973		
5		+++++	20.911	148.45	14.086		
6	*Scenedesmus* (?) sp.	(++)	20.310	148.70	13.658	13.784	−0.678
6		(++)	20.799	148.60	13.997		
6		(++)	20.366	148.70	13.696		
11	*Chlorella* (?) sp.	(++++)	20.212	148.90	13.574	13.834	−0.628
11		(++++)	21.162	148.95	14.208		
11		(++++)	20.450	149.05	13.720		
7	*Protococcus* sp.	+++++	21.036	148.95	14.123	14.032	−0.430
7		+++++	20.771	149.00	13.940		
3	*Protosiphon*	+++	22.098	148.95	14.836	14.398	−0.064
3	*botryoides*	+++	20.827	149.20	13.959		
	Average all cultures		.....	.....	.....	14.033	−0.429

7B. *Same nitrogen source. Medium with 1 percent mannite*

—	Check	Ster.	20.520	148.55	13.814	13.993	
—	Check	Ster.	21.162	148.80	14.222		
—	Check	Ster.	13.872	99.50	13.942		
1	*Chlorella vulgaris* Beyr.	+++*	20.966	148.35	14.133	14.321	+0.328
1		+++	22.070	148.40	14.872		
1		+++	20.729	148.50	13.959		
2	*Stichococcus* sp.	++	20.938	148.65	14.085	13.658	−0.335
2		++	19.570	148.70	13.161		
2		++	20.408	148.65	13.729		
5	*Chlorella* (?) sp.	+++	21.064	148.50	14.185	13.780	−0.213
5		+++	20.603	148.70	13.855		
5		+++	19.765	148.60	13.301		
6	*Scenedesmus* (?) sp.	++	21.637	148.80	14.541	14.061	+0.068
6		++	20.087	148.70	13.508		
6		++	21.008	148.65	14.133		
11	*Chlorella* (?) sp.	++	19.905	148.80	13.377	13.584	−0.409
11		++	20.701	148.90	13.903		
11		++	20.073	149.00	13.472		
7	*Protococcus* sp.	+	20.226	149.00	13.575	13.627	−0.366
7		+	20.380	149.00	13.678		
3	*Protosiphon botryoides*	+++	20.757	149.15	13.917	14.040	+0.047
3		+++	21.134	149.20	14.162		
	Average all cultures		.....	.....	.....	13.867	−0.126

TABLES 8, 8A, AND 8B. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1919. Growth period, 255 days. Nitrogen source,*

AMMONIUM NITRATE

8. *Medium without glucose or mannite*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
—	Check	Ster.	26.704	147.45	18.111	17.736	
—	Check	Ster.	26.789	147.95	18.107		
—	Check	Ster.	25.189	148.25	16.991		
1	*Chlorella vulgaris* Beyr.	+++++*	27.015	147.65	18.292	18.308	+0.672
1		+++	27.029	147.25	18.356		
1		+++	26.958	147.50	18.277		
2	*Stichococcus* sp.	++*	26.576	147.35	18.036	17.989	+0.253
2		+	26.166	147.50	17.740		
2		+	26.888	147.80	18.192		
5	*Chlorella* (?) sp.	+++	25.543	147.65	17.300	17.198	−0.538
5		++	25.413	147.90	17.183		
5		++	25.274	147.70	17.112		
6	*Scenedesmus* (?) sp.	+++	26.619	147.75	18.016	17.866	+0.130
6		+++	26.194	147.95	17.705		
6		+++	26.421	147.80	17.876		
11	*Chlorella* (?) sp.	+++	20.248	147.90	13.690	14.018	−3.718
11		+++	20.615	147.95	13.934		
11		++++	21.380	148.15	14.431		
	Average all cultures (omitting species no. 11)		.....	.....	.....	17.840	+0.104

8A. *Same nitrogen source, medium with 1 percent glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.	Percent Gain
No.	Species							
—	Check	Ster.	25.095	147.70	16.991	17.178		
—	Check	Ster.	25.499	148.00	17.229			
—	Check	Con.*	25.529	147.45	17.314			
1	*Chlorella vulgaris*	(+)	26.774	147.55	18.145	18.272	+1.094	6.
1	Beyr.	(+)	27.369	147.50	18.555			
1		(+)*	26.774	147.80	18.115			
2	*Stichococcus* sp.	(++)	26.180	147.30	17.773	17.417	+0.239	1.4
2		(++)	23.009	147.15	15.636			
2		(++)*	27.822	147.65	18.843			
5	*Chlorella* (?) sp.	+++++	27.161	147.20	18.452	18.973	+1.795	10.
5		+++++	28.658	147.75	19.396			
5		+++++*	28.195	147.85	19.070			
6	*Scenedesmus* (?) sp.	+++++	27.496	147.80	18.604	17.911	+0.733	4.2
6		+++++	24.452	147.85	16.538			
6		+++++	27.496	147.90	18.591			
11	*Chlorella* (?) sp.	+++++	27.358	148.10	18.473	18.293	+1.115	6.5
11		+++++	27.037	148.15	18.250			
11		+++++	26.897	148.15	18.155			
7	*Protococcus* sp.	+++++	28.377	148.10	19.161	19.303	+2.125	12.
7		+++++	28.992	148.25	19.556			
7		+++++	28.433	148.15	19.192			
3	*Protosiphon*	+++	Lost	148.35	.....	18.071	+0.893	5.2
3	*botryoides*	+++	26.619	147.30	18.071			
	Average all cultures		.....	.....	.....	18.320	+1.142	6.6

8B. *Same nitrogen source, medium with 1 percent mannite*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
—	Check	Ster.	25.741	146.95	17.517	17.091	
—	Check	Ster.	25.571	148.10	17.266		
—	Check	Con.*	24.184	146.65	16.491		
1	*Chlorella vulgaris*	+++	25.458	146.65	17.360	17.587	+0.496
1	Beyr.	+++	26.350	146.65	17.968		
1		+++	25.033	143.60	17.433		
2	*Stichococcus* sp.	++	24.467	146.80	16.667	16.466	−0.625
2		++	24.000	146.90	16.338		
2		++	24.113	147.10	16.392		
5	*Chlorella* (?) sp.	+++	26.746	147.10	18.182	17.243	+0.152
5		+++	24.368	147.15	16.560		
5		+++	25.047	147.45	16.987		
6	*Scenedesmus* (?) sp.	+++	26.307	147.50	17.835	17.403	+0.312
6		+++	24.269	147.55	16.448		
6		+++	26.449	147.55	17.926		
11	*Chlorella* (?) sp.	++	21.933	147.75	14.845	14.891	−2.200
11		++	22.258	147.80	15.060		
11		++	21.848	147.95	14.767		
7	*Protococcus* sp.	+	24.807	147.85	16.779	17.114	+0.023
7		+	25.840	148.10	17.448		
3	*Protosiphon*	++	25.812	148.15	17.423	16.812	−0.279
3	*botryoides*	++	24.099	148.20	16.200		
	Average all cultures (omitting species no. 11)		.....	.....	.....	17.103	+0.012

TABLES 9, 9A, AND 9B. *Results of analyses of mineral nutrient agar cultures of green algae. Experiment of 1919. Growth period, 235 days. Nitrogen source,* CALCIUM NITRATE

9. *Medium without glucose*

Culture		Growth	Total Mg. N Found	Wt. of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.	Gain Percent
No.	Species							
—	Check	Ster.	24.202	147.90	16.364	16.755		
—	Check	Ster.	24.146	147.95	16.320			
—	Check	Ster.	26.045	148.15	17.580			
1	*Chlorella vulgaris*	+++	25.822	147.25	17.536	17.874	+1.119	6.
1	Beyr.	+++	26.939	147.15	18.307			
1		+++	26.199	147.35	17.780			
2	*Stichococcus* sp.	+	22.735	147.30	15.435	17.058	+0.303	
2		++	26.464	147.45	17.948			
2		+	26.241	147.50	17.791			
5	*Chlorella* (?) sp.	++	27.322	147.60	18.511	18.689	+1.934	11.5
5		++	26.520	147.90	17.931			
5		++	28.978	147.65	19.626			
6	*Scenedesmus* (?) sp.	++	22.875	147.85	15.472	16.522	−0.233	
6		++	24.341	147.90	16.458			
6		++	26.073	147.85	17.635			
11	*Chlorella* (?) sp.	+++	17.121	148.25	11.549	11.439	−5.316	
11		+++	17.749	148.15	11.980			
11		+++	16.046	148.75	10.787			
	Average all cultures (omitting species no. 11)		.....	.....	.....	17.311	+0.556	

9A. *Same nitrogen source, medium with 1 percent glucose*

—	Check	Ster.	23.055	148.70	15.504	15.640		
—	Check	Ster.	22.553	148.85	15.152			
—	Check	Ster.	24.298	149.40	16.264			
1	*Chlorella vulgaris*	+++++	34.535	148.70	23.225	22.963	+7.323	47.
1	Beyr.	+++++*	33.948	148.50	22.861			
1		+++++	33.864	148.50	22.804			
2	*Stichococcus* sp.	++++++	35.024	148.50	23.585	22.440	+6.800	43.5
2		++++++	30.820	148.50	20.754			
2		++++++	34.186	148.75	22.982			
5	*Chlorella* (?) sp.	+++++	34.849	148.70	23.436	23.677	+8.037	51.4
5		+++++	35.799	148.90	24.042			
5		+++++	35.071	148.90	23.553			
6	*Scenedesmus* (?) sp.	+++++	34.388	148.75	23.118	23.434	+7.794	49.7
6		+++++	34.975	149.15	23.450			
6		+++++	35.338	148.90	23.733			
11	*Chlorella* (?) sp.	++++++	31.714	148.90	21.299	21.319	+5.679	36.3
11		++++++	32.831	149.00	22.034			
11		++++++	30.750	149.10	20.624			
7	*Protococcus* sp.	+	24.243	149.05	16.265	16.372	+0.732	4.7
7		+	24.578	149.15	16.479			
3	*Protosiphon*	++++	25.360	149.15	17.003	17.372	+1.732	11.
3	*botryoides*	++++	26.477	149.25	17.740			
	Average all cultures		.....	.....	.....	21.082	+5.442	34.8

9B. *Same nitrogen source, medium with 1 percent mannite*

Culture		Growth	Total Mg. N Found	Weight of Medium, Grams	Mg. N per 100 Gr. Medium	Average	Mg. Loss or Gain, 100 Gr.
No.	Species						
—	Check	Ster.	27.865	148.75	18.733	17.728	
—	Check	Ster.	25.360	148.55	17.071		
—	Check	Con.*	25.826	148.60	17.380		
1	*Chlorella vulgaris*	+++	22.818	148.70	15.345	15.280	−2.448
1	Beyr.	+++	23.558	148.90	15.821		
1		+++	21.799	148.55	14.675		
2	*Stichococcus* sp.	+++	21.904	148.70	14.730	16.529	−1.199
2		++	24.311	148.70	16.349		
2		++	27.511	148.65	18.507		
5	*Chlorella* (?) sp.	++	26.973	148.60	18.151	17.764	+0.036
5		++	27.029	148.60	18.189		
5		++	25.189	148.60	16.951		
6	*Scenedesmus* (?) sp.	++	19.634	148.55	13.217	15.603	−2.125
6		++	24.326	148.65	16.365		
6		++	25.609	148.65	17.228		
11	*Chlorella* (?) sp.	+++	13.481	148.70	9.066	9.430	−8.298
11		+++	Lost	148.80	.....		
11		+++	14.613	149.20	9.794		
7	*Protococcus* sp.	+	22.251	149.20	14.914	14.914	−2.814
7		+	Lost	149.15	.....		
3	*Protosiphon*	+++	24.481	149.15	16.414	17.063	−0.665
3	*botryoides*	+++	26.293	148.45	17.712		
	Average all cultures (omitting species no. 11)		.....	.....	.....	16.192	−1.536

Discussion

From a study of the foregoing tables it is apparent at once that substantial increases in the total combined nitrogen content of the culture flasks occurred with the media containing nitrate nitrogen and glucose. Fixation on these media was not confined to any one species, most of the forms used showing the ability to utilize the uncombined nitrogen of the air. The cases of Protococcus (species no. 7) and Protosiphon (species no. 3) may be questioned, but it will be noticed that of all the species grown on ammonium nitrate with glucose in the 1919 experiment (table 8A) Protococcus gave the highest gain in nitrogen, and that in the same experiment Protosiphon gave an increase of nearly 2 mg. on calcium nitrate with glucose (table 9A). Although an increase of only 2 mg. is a small amount to base definite conclusions upon, the evidence is certainly in favor of the assumption that these two species also possess the ability to fix nitrogen. As regards species nos. 1, 2, 5, and 6 there can be no question about their ability to fix nitrogen, since the increases with these species range from a fraction of a milligram to over 8 mg. per 100 grams medium, representing additions to the total nitrogen content of the flasks of from 1 to 51 percent. The highest increase noted was in the case of one of the cultures of species no. 5 on calcium nitrate with glucose, in the 1919 experiment (table 9A); this flask showed a total gain of 12.53 mg. over the average of the checks, an increase in nitrogen content of 54 percent. Fixation was secured by these four species in both experiments when grown on similar media and under similar conditions, so that there seems to be no escape from the definite conclusion that these forms do actually use free nitrogen. The amounts of fixation secured by these four species on ammonium nitrate with glucose in 1919 were considerably lower than found in the 1917–18 experiment on a similar medium. As already pointed out, however (page 9), the growth produced on this medium in 1919 was very poor, a factor which, it is believed, accounts for the smaller fixation.

This leaves to be considered the case of species no. 11 (*Chlorella* sp.) which is of considerable interest. In series 8A and 9A (the nitrate media with glucose) substantial increases in the nitrogen content of the cultures occurred, especially on calcium nitrate, where the average of the cultures is more than 5.5 mg. above the checks. This same species, however, on both nitrate media without a carbon source, and on the nitrate media with mannite, showed distinct losses in nitrogen. These losses ranged from 2 to 8 mg. and occurred regularly on the media mentioned with this species only. This species was used only in the 1919 experiment, in which acid tubes were not inserted between adjacent culture flasks, so that there is no means of telling whether the loss occurred as ammonia or as free nitrogen. However, that some sort of process resulting in a loss of introgen has taken place is apparent, and the results suggest that perhaps both the processes of free nitrogen fixation and of denitrification may be going on simultaneously, the former overbalancing the latter when glucose is present.

As regards the use of free nitrogen by the forms investigated on nitrate media to which no carbohydrate energy source was added, the evidence is not conclusive. In 1917–18 gains of from 0.7 to 1.8 mg. per 100 grams medium were recorded for three of the species grown on ammonium nitrate (table 4), and one of these species gave an increase of 1.8 mg. on calcium nitrate without a carbon source (table 5). In 1919 three out of five species showed very slight increases on ammonium nitrate in the absence of a carbon source, while on calcium nitrate without glucose or mannite gains of from 0.3 to 1.9 mg. per 100 grams medium occurred with three species (table 9). As has already been pointed out, however, the growth of all species in the absence of glucose is very slow, and, although the cultures remain in a healthy condition for long periods of time, the actual amount of "crop" produced is slight in comparison with the glucose cultures. That some fixation does take place in the absence of glucose seems probable, in view of the increases cited above, and the assumption that the amount of fixation on one and the same medium is dependent, more or less, on the total amount of growth produced does not seem unwarranted. This phase of the problem is of considerable importance and is one which should be most carefully investigated, as it bears directly on the conditions as they exist in nature. It is entirely possible that the conditions realized in the experiments, under which marked fixation occurred, have merely amplified those already existing in the field, for soluble carbohydrates and a certain amount of nitrates would most certainly be available for the development of these organisms, especially those occurring naturally on soil. If this is actually the case, it is highly probable that fixation occurs also under natural conditions.

The evidence that no fixation occurred on media lacking nitrates is quite conclusive. When nitrogen was supplied as glycocoll, asparagine, urea, or ammonium sulphate, the increase or decrease in the nitrogen content of the culture flasks above or below that of the checks was in all cases less than a milligram, even though the growth, especially on urea with glucose, was as luxuriant as that produced on nitrates in the presence of glucose. The results were uniform for all species grown on media containing these nitrogen sources and were not altered by the presence either of glucose or of mannite, the latter being used only with ammonium sulphate. Although some of the cultures of these series showed higher nitrogen contents than the checks, there were no consistent gains, and in most cases the figures were lower than those of the checks. However, the differences in either case are so small as to be without significance, since most of them are within the usual experimental error.

A comparison of the results presented here with figures secured by a large number of investigators working with the legume nodule bacteria and Azotobacter forms shows that the amount of fixation produced by the algae per unit volume of medium, under the conditions of the experiments, equals, and in some cases surpasses, the average fixation by the colorless forms. The legume bacteria especially give only slight increases when grown in pure culture outside the host plant. Fred (1912) gives a table of the results of 25 investigators, from which it is apparent that the average fixation with this organism is about 0.9 to 2.2 mg. per 100 cc. culture medium. Fred himself secured fixations ranging from 0.4 to 1.58 mg. per 100 cc. culture solution. With the Azotobacter forms the amounts of fixation are considerably larger. *Azotobacter chroococcum*, which has been extensively investigated in pure culture, shows increases ranging from a few milligrams to 20 or 30 mg. per 100 cc. culture medium, the average fixation, however, being usually about 6 or 10 mg. Thus Gerlach and Vogel (1902) report 4 mg. to be the average, while Freudenreich (1903) secured only 1.6 to 2.4 mg. per 100 cc. culture solution, but on gypsum plates the increases were higher, amounting to 16 mg. per 100 cc. solution. Krzemieniewski (1908) secured increases ranging from 1 to 13.5 mg. per 100 cc. solution, an average of 3 to 6 mg. being fixed for each gram of glucose respired. In the presence of humus the ratio was 5 to 10 mg. nitrogen fixed per gram of glucose respired. Hoffmann and Hammer (1910) report fixations of from 4.55 to 14.4 mg. per gram of carbohydrate consumed, and in the same year Krzemieniewska (1910) published results showing increases of from 11 to 18 mg. per 100 cc. culture solution. Remy and Rosing (1911) found from 10 to 20 mg. to be the average fixation per 100 cc. culture, or 2 to 15 mg. per gram of mannite consumed. Perhaps the most extensive experiments with Azotobacter forms are those of Lipman (1903 and 1905), who secured the following gains in nitrogen, each in 100 cc. of culture solution: *A. chroococcum*, 4.42 mg.; *A. beyerinckii*, 2.37 to 6.78 mg.; *A. vinlandii*, 1.67 to 7.90 mg. in 1903, and 8.36 to 20.99 mg. in 1905. The numerous results with mixed cultures and impure soil cultures by many investigators show even wider ranges of fixation, and the conditions of the experiments have been so various that it is difficult to arrive at an average, but in many cases it appears to be between 6 and 10 mg. per 100 grams of culture medium.

Because of the nature of the experiments reported in this paper it is impossible to state in what form the nitrogen is added to the culture. Whether or not the process is connected with that of photosynthesis, the ratio of nitrogen fixed to sugar respired or to the amounts of nitrate absorbed, and the amount fixed per unit dry weight of crop produced are phases of the problem which can be merely mentioned at this time. The solution of these questions will be simplified by the proper development of liquid culture methods, some of which are now in progress.

Since the results obtained are wholly contrary to the generally accepted idea of the relation of green plants to elementary nitrogen, it may be well to point out the conditions under which some of the earlier negative results with the green algae were obtained. The first nitrogen determinations of pure cultures of algae were made by Kossowitsch (1894), who grew a single species of Cystococcus (?) on sand moistened with 20 cc. of a mineral nutrient solution containing calcium nitrate as a source of nitrogen. The cultures, however, grew for only three weeks, because, the author concludes, of the lack of nitrates, only 2.5 mg. nitrogen as nitrate having been supplied. The addition of more calcium nitrate to the cultures caused them to revive, and it was upon this fact that his conclusion was based. However, it is not altogether clear that the lack of growth may not have been due to some other cause, such as deficiency in calcium, only a "trace" of which was present in addition to that added as calcium nitrate. The analyses of the cultures and uninoculated checks clearly showed that there had been no increase in the nitrogen content of the flasks, either in the presence or in the absence of glucose. It is apparent, however, that conditions for a vigorous, long-continued growth were not realized in these experiments, hence it is not to be expected that appreciable increases in nitrogen should occur. As a result of a large number of analyses of pure cultures, Kruger and Schneidewind (1900) concluded that the green algae investigated were unable to use free nitrogen. They grew a large number of forms on a variety of media, using both sand and solution cultures. Two of the media contained nutrient solutions free from combined nitrogen, five contained organic nitrogen supplied in beef extract and peptone, beerwort, or humus, and in the two remaining solutions both ammonium sulphate and sodium nitrate were present; in none of the media was the nitrogen supplied as nitrate only. The cultures were grown in large flasks plugged with cotton, no other provision being made for aeration. No growth resulted on the media free from combined nitrogen; on those to which organic nitrogen sources were added, a vigorous development of the algae resulted in most cases but no increases in nitrogen content were obtained. On the media containing inorganic nitrogen a good growth was obtained only in one experiment with cultures of several species of Stichococcus; in the remaining experiments with these media there was no growth because of the unfavorable reaction of the solution. No increases in nitrogen content were observed, however, in any case. The results of these authors compare very favorably with those presented above in the tables, no fixation having been secured when ammonium sulphate or organic compounds served as nitrogen sources.

In 1903, Charpentier substantiated Kossowitsch's results with Cystococcus. He grew the single species on a bean-glucose-gelatin medium; at the end of 20 days no change in the nitrogen content of the cultures was observed. However, these experiments were neither extensive enough, nor were they continued for sufficient length of time, to be conclusive. The failure to secure fixation may be attributed in this case also to the absence of proper nitrogen sources in the medium.

As already pointed out in the first part of this discussion, the presence of nitrate nitrogen seems to be essential for the process of fixation. In this connection it is interesting to note that the presence of nitrates has been found beneficial for the growth of, and fixation of nitrogen by, Azotobacter forms and the legume bacteria. It has been repeatedly observed that the presence of very slight amounts of nitrates stimulates the development of the nodule bacteria. Thus, Fred and Graul (1916) report that in the presence of small amounts of ammonium nitrate nodules were produced in great numbers on alfalfa and vetch by pure cultures of *Bacillus radicicola*; numerous nodules were formed also in the presence of small amounts of calcium nitrate, but with added amounts the degree of infection declined. In a recent paper, Hills (1918) reports that the presence of small amounts of nitrates exerted an enormous influence on the growth and reproduction of Azotobacter in sterile soil, the amount of fixation in such cultures being also increased in the presence of nitrates. In the case of *Bacillus radicicola* the nitrates stimulated growth and reproduction in sterilized soil, and on agar films the amount of fixation was greater when nitrates were added. The author concludes that this increased fixation may be the result of the increased growth of the organisms where nitrates were supplied. Though such observations as these cannot be claimed to have any direct application to the case of green plants, they are at least suggestive in view of the fact that nitrates have long been regarded as in general the best combined nitrogen source for chlorophyll-bearing plants.

The proof that green plants are capable of fixing free nitrogen is at once of real significance, both from a purely scientific and from a practical standpoint, even though the plants concerned are members of a low order. The question which perhaps at first suggests itself is: Do all green plants possess this ability to use the uncombined nitrogen of the air? With the exception of the legumes, the evidence is almost entirely negative as regards the flowering plants. Molliard (1916) has recently demonstrated that radish plants in pure culture are unable to increase the nitrogen content of the culture. The plants were grown in large tubes on nutrient solutions containing several different concentrations of ammonium chloride as a source of nitrogen, some cultures being supplied with glucose, and others being aerated with air charged with carbon dioxide in order to increase the photosynthetic activity. In no case did the total nitrogen of the crop and residual culture solution exceed that of the seed and original solution, even after six weeks' growth. The plants produced, however, were small, bearing three or four leaves in addition to the cotyledons, the green weight varying from 0.3 to 1.3 gr. Moreover, nitrate nitrogen was not supplied as a source of nitrogen in any case, a fact which may be of considerable importance in view of the results presented in this paper. It is possible that in the case of the higher plants also the proper culture conditions have not yet been realized, since in the case of four radish plants grown with the roots under aseptic conditions and the tops in free air, gains of 1.32 mg. nitrogen per plant were reported by Molliard, but he assumes that this nitrogen has come from the ammonia of the air.

The significance of the legume bacteria has recently been questioned by Beijerinck (1918) who argues that, with the exception of lupine and seradella, the number and weight of the tubercles is insignificant in comparison with the total weight of the plant. The isolated organisms fix nitrogen in pure culture only very slightly or not at all, and no fixation could be demonstrated with nodules placed in glass jars in which the changes of gases were measured. In the case of Robinia the number of tubercles is extremely small and fixation in them must be very intense, yet when tested they were found to be inactive or nearly so. He concludes that "the present accepted explanation of the behavior of legumes cannot be correct." The situation in the higher plants needs to be more carefully and thoroughly investigated.

CONCLUSIONS

1. Seven species of grass-green algae (Chlorophyceae) exhibited the ability to fix nitrogen when grown in pure cultures on mineral nutrient agar media containing either ammonium nitrate or calcium nitrate as a souce of nitrogen, and glucose. The nitrogen fixed was derived from the free (uncombined) nitrogen of the atmosphere. The amounts of fixation ranged

from 1 to 12.5 mg., representing increases in the total nitrogen content of the culture flasks of from 4 to 54 percent.

2. Five of the above mentioned species were grown on ammonium nitrate and calcium nitrate in the absence of glucose. Four of these showed slight increases in nitrogen over the original content of the medium; growth in the absence of glucose, however, was slight.

3. There was no fixation when urea, glycocoll, asparagine, or ammonium sulphate was supplied as a nitrogen source, either in the presence or in the absence of glucose or of mannite.

4. One species (no. 11) exhibited what is apparently a denitrification on media containing either ammonium nitrate or calcium nitrate as nitrogen sources, both in the absence of an organic carbon source and in the presence of mannite. The loss in nitrogen amounted to from 2.2 to 8.3 mg. per 100 grams culture medium.

Acknowledgments

The writer wishes to express his sincere appreciation to Dr. J. R. Schramm, whose many helpful suggestions and constant interest have been invaluable aids in the completion of the experiments, to Dr. L. W. Sharp for the photographic work, and to other members of the department for kindly assistance in many ways.

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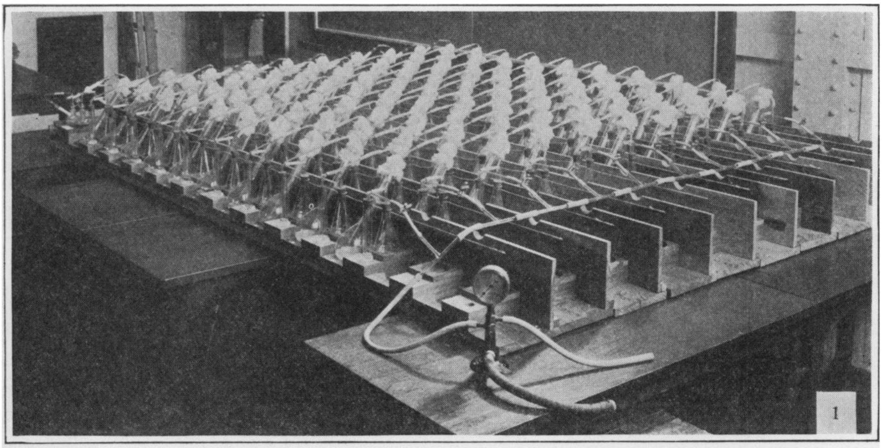

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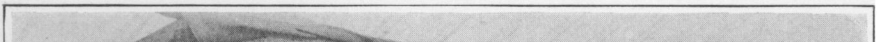

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EXPLANATION OF PLATE I

FIG. 1. The complete apparatus of the 1917–18 experiment. The ten series are connected, on the right, by T-tubes, forming a single complete series for the purpose of aeration.

FIG. 2. The complete apparatus of the 1919 experiment, occupying a bench in the greenhouse. The series in the foreground is no. 6A (urea, with glucose), on which a very vigorous growth was produced by all species except no. 7 (*Protococcus* sp.); the fourth and fifth flasks from the right of the series were inoculated with this species. The check flasks (7th, 14th, and last from the left) stand out clearly because of the absence of growth in them.